BIOSERVICE
SCIENTIFIC LABORATORIES GmbH

Mammalian Micronucleus Test of Murine Bone Marrow Cells

with

WACKER BS 1701

Final Report

BSL BIOSERVICE Project No.: 001702

Sponsor:

Wacker-Chemie GmbH

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Bescheinigung

Hiermit wird bestätigt, daß die Prüfeinrichtung(en)

BSL Bioservice Scientific Laboratories GmbH
in 82152 Planegg (Ort, Anschrift)
Behringstraße 6
der Firma BSL Bioservice Scientific Laboratories GmbH (Firma)
am 29./30. November 1999 (Datum)

von der für die Überwachung zuständigen Behörde über Einhaltung der Grundsätze der Guten Laborpraxis inspiziert worden ist (sind).

Es wird hiermit bestätigt, daß folgende Prüfungen in dieser Prüfeinrichtung nach den Grundsätzen der Guten Laborpraxis durchgeführt werden.

Certificate

It is hereby certified that the test facility(ies)

BSL Bioservice Scientific Laboratories GmbH
in 82152 Planegg (location, address)
Behringstraße 6
of Firma BSL Bioservice Scientific Laboratories GmbH (company name)
on 29./30. November 1999 (date)

was (were) inspected by the competent authority regarding compliance with the Principles of Good Laboratory Practice.

It is hereby certified that studies in this test facility are conducted in compliance with the Principles of Good Laboratory Practice.

Die Prüfungen von Stoffen und Zubereitungen betreffen folgende OECD-Prüfkategorie

Prüfkategorie 2: Prüfungen auf toxikologische Eigenschaften

Prüfkategorie 3: Prüfungen auf mutagene Eigenschaften (in vitro, in vivo)

Prüfkategorie 9: Sonstige Prüfungen; a) Mikrobiologische Sicherheitsprüfungen
b) Wirksamkeitsprüfungen an Zellkulturen

München, 04.08.2000
I.V.

Ritter
Leitender Gewerbedirektor

L.S.

Contents

Preface

General

Sponsor:	Wacker-Chemie GmbH Werk Burghausen Johannes-Hess-Strasse 24 D-84489 Burghausen, Germany
Monitor:	Dr. Axel Bosch
Testing Facility:	BSL BIOSERVICE Scientific Laboratories GmbH Behringstrasse 6 D-82152 Planegg/München
BSL BIOSERVICE - Project No.:	001702
Test Item:	WACKER BS 1701
Title:	Mammalian Micronucleus Test of Murine Bone Marrow Cells with WACKER BS 1701

Project Staff

Study Director:	Dipl. Biol. Uwe Hamann
Responsible Veterinarian:	Dr. Achim Albrecht
Deputy Director of the Testing Facility:	Dr. Angela Lutterbach
Quality Assurance Unit:	Dr. Margarete Hoechst Dipl. Biol. Maike Führböter

Schedule

Arrival of the Test Item:	October 26, 2000
Date of Project Protocol:	October 30, 2000
Start of Experiments:	November 27, 2000
End of Experiments:	January 15, 2001
Date of Draft Report:	February 06, 2001
Date of Final Report:	February 26, 2001

Project Staff Signatures

Study Director: Dipl. Biol. Uwe Hamann

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Date: 26.02.2001

Deputy Director of
the Testing Facility : Dr. Angela Lutterbach

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Date: 26.02.2001

Quality Assurance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated August 01, 1994 (BGBL. I, 1994, S. 1703).

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998.

This study is assessed in compliance with the project protocol, the study plan and the Standard Operation Procedures of BSL BIOSERVICE. The study and/or the testing facility are periodically inspected by the Quality Assurance Unit and the dates and phases of the inspections are included in the report. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The final report of the study is audited. A Quality Assurance Statement, signed by the Quality Assurance, is included in the report.

Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 474 "Micronucleus Test", adopted July 21, 1997.

EEC Directive 2000/32, L 136, Annex 4C, B 12, dated June 08, 2000.

Archiving

The following records will be stored in the scientific archives of BSL BIOSERVICE Scientific Laboratories GmbH according to the GLP-regulations:

a copy of the final report, the project protocol, the study plan and a documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, microscopic slides, printouts of instruments and computers) and the correspondence with the sponsor concerning the project.

The microscopic slides and a sample of the test item (if left over) will be stored according to the period fixed by the GLP-regulations. Samples that are unstable my be disposed off before that time. Unless otherwise agreed upon, remaining test item will be discarded three months after release of the final report.

No raw data or material relating to the study will be discarded without the sponsor's prior consent.

Statement of Compliance

BSL BIOSERVICE

Project-No.: 001702

Test Item: WACKER BS 1701

Study Director: Dipl. Biol. Uwe Hamann

Title: Mammalian Micronucleus Test of Murine Bone Marrow Cells with WACKER BS 1701

This study performed in the testing facilities of BSL BIOSERVICE Scientific Laboratories GmbH was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated August 01, 1994 (BGBL. I, 1994, S. 1703).

OECD Principles of Good Laboratory Practice (as revised in 1997); Paris 1998.

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Dipl. Biol. Uwe Hamann

Date: 28.02.2001

Quality Assurance Unit

BSL BIOSERVICE Scientific Laboratories GmbH
Behringstr. 6, D-82152 Planegg

Statement

BSL BIOSERVICE

Project-No.:	001702
Test Item:	WACKER BS 1701
Study Director:	Dipl. Biol. Uwe Hamann
Title:	Mammalian Micronucleus Test of Murine Bone Marrow Cells with WACKER BS 1701

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates:

	Phases and Dates of QAU Inspections	Dates of Reports to the Study Director and Management
Audit Project Protocol/ Study Plan:	November 06, 2000	November 06, 2000
Experimental Phase Audit (Method Audit):	January 19, 2000	January 19, 2000
Draft Audit:	February 14, 2001	February 14, 2001
Final Audit:	February 28, 2001	February 28, 2001

This report reflects the raw data.

Head of Quality Assurance Dr. Margarete Hoechst (or)
Dipl. Biol. Maike Führböter

Margarete Hoechst

Date: 28.02.2001

Summary

This study was performed to investigate the potential of WACKER BS 1701 to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse.

The test item was prepared in carboxy methyl cellulose (CMC) 1 %. The volume administered orally was 10 ml/kg b.w.. 24 and 48 h after a single application of the test item the bone marrow cells were collected for micronuclei analysis.

Ten animals (5 males, 5 females) per test group were evaluated for the occurrence of micronuclei. 2000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei.

To describe a cytotoxic effect due to the treatment with the test item the ratio between polychromatic and normochromatic erythrocytes (NCE) was determined in the same sample and reported as the number of NCE per 1000 PCE.

The following dose levels of the test item were investigated:

24 h preparation interval: 200, 1000 and 2000 mg/kg b.w.

48 h preparation interval: 2000 mg/kg b.w.

In a pre-experiment with 2000 mg/kg b.w. the animals expressed no toxic reactions after orally administration. Therefore the maximum dose in the main experiment was 2000 mg/kg b.w..

The ratio of normochromatic to polychromatic erythrocytes was not affected by the treatment with WACKER BS 1701 at a dose of 2000 mg/kg b.w. at 24 hours and slightly effected after 48 treatment indicating that the test item had no or only slight toxic properties.

In comparison with the corresponding negative controls there was no substantial enhancement in the frequency of the detected micronuclei at any dose level of the test item.

An appropriate reference mutagen was used as positive control which showed a distinct increase of induced micronucleus frequency.

Conclusion

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the mouse.

Therefore, WACKER BS 1701 is considered to be non-mutagenic in this micronucleus assay.

Objective

Aims of the Study

This *in vivo* experiment was performed to assess the mutagenic properties of the test item by means of the micronucleus test in bone marrow cells of the mouse.

Reasons for the Study

The occurrence of micronuclei in interphase cells provides an indirect but easy and rapid measure of chromosomal damage. Micronuclei arise from acentric chromosomal fragments or whole chromosomes induced by clastogens or agents affecting the spindle apparatus (1,2,3,4).

Polychromatic erythrocytes (PCE) in the bone marrow of the mouse are the cell population of choice for mammalian cells *in vivo*. PCEs are newly formed red blood cells and are easily identifiable by their staining properties. These cells have the advantage that the micronuclei can be readily detected because the nucleus is extruded from the erythroblast after the last cell division.

The first appearance of micronuclei in PCEs is at least 10-12 hours after a clastogenic exposure. This lag is due to the time required for the affected erythroblast to differentiate into a PCE. This differentiation process includes:

1.The time required for the damaged erythroblast to proceed to mitosis.

2.The mitotic delay induced by the treatment.

3.The formation of micronuclei due to acentric fragments or chromosomes that are not included in the daughter nuclei.

4.The time required for the expulsion of the main nucleus after the last mitosis to become a micronucleated PCE.

This newly formed cell population persists for about 20 hours in the bone marrow of mouse. During this time micronucleated PCEs accumulate in the bone marrow as the production of micronuclei extends over a considerable period of time.

For the assessment of clastogenic activity at least 3 dose levels are applied, of which the highest dose is either the maximum tolerated dose or that producing some indication of cytotoxicity (change in the ratio of polychromatic to normochromatic erythrocytes) and sampling is carried out 24h and 48 hours after treatment. To validate the test, a reference mutagen is tested in parallel to the test item.

Materials and Methods

Characterisation of the Test Item

The test item and the information concerning the test item were provided by the sponsor.

Name:	WACKER BS 1701
Chemical description:	Alkylalkoxysilane
Batch No.:	KH 02343
CAS.-No.:	35435-21-3
Aggregate State at RT:	liquid
Colour:	colourless
Density (g/cm³):	0.86 at 25°C
Structural Formula:	not provided
Purity:	98.53%
Analysis:	GC
Stability:	Pure: years
	Stable in aqueous solution for at least 24 hours
Storage:	at room temperature, protected from light
Expiry Date:	November 2001
Safety Precautions:	Routine hygienic procedures will be sufficient to assure personnel health and safety.

The test item was prepared and diluted with 1 % CMC (Carboxymethyl-cellulose, SIGMA, Lot No: 36H0738). All animals received a single standard volume p. o. of 10.0 ml/kg b.w.. The vehicle was chosen according to its relatively nontoxicity for the animals.

The Controls

Positive and negative controls were included.

The Negative Control

The vehicle of the test item 1 % CMC was used as negative control. All control animals were handled in an identical manner to the test group subjects.

The Positive Control

Name	CPA; Cyclophosphamide
Supplier	SIGMA, D-82039 Deisenhofen
Catalogue no.	C0768 (purity: at least 98 %)
Lot no.	087H0207
Dissolved in	0.9% NaCl
Final concentration	40 mg/kg b.w.
Route and frequency of administration	i.p., single
Volume administered	10 ml/kg b.w.

The solution was prepared on day of administration. The stability of CPA at room temperature is quite good (1% is hydrolysed per day in aqueous solution).

The sampling time for the controls was 24 hours.

The Test System

The animals

The mouse is an animal which has been used for many years as suitable experimental animal in cytogenetic investigations. There are an abundance of available data, which may aid the interpretation of the results of the micronucleus test. In addition, the mouse is an experimental animal in many physiological, pharmacological and toxicological studies. Data from such experiments may also be useful for the design and the performance of the micronucleus test (1,2,3,4,5).

Strain	NMRI
Source	HARLAN WINKELMAN (D-33178, Borchen)
Number of animals	5 of each sex per dose group
Initial body weight at the start of experiment	24 - 42 g

According to the suppliers assurance the animals were in a healthy condition. The animals were under quarantine in the animal facilities of BSL BIOSERVICE for a minimum of 5 days after arrival. During this period the animals did not show any signs of illness or altered behaviour.

Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions.

Housing	5 animals of identical sex per cage
Cage type	Macrolon Type III (Hereto, D-79302 Emmendingen)
Bedding	granulated soft wood bedding (ALTROMIN, D-32791 Lage/Lippe)
Feed	pelleted standard diet (ALTROMIN, D-32791 Lage/Lippe)
Water	tap water, ad libitum (Zweckverband Würmtal, D-82152, Planegg)
Environment	temperature 19-25° C relative humidity 55±10% artificial light 6:00-18:00

The animals were individually marked for identification by tail drawing.

Experimental Performance

Pre-experiment for Toxicity

A preliminary study on acute toxicity was performed with the same strain and under identical conditions as in the mutagenic study.

According to the used guidelines the highest applicable dose is 2000 mg/kg b.w.. In the initial step 2000 mg/kg b.w. were administered orally where no toxic signs were found. Due to this result 2000 mg/kg b.w. was tested as the maximum dose in the main experiment. No further investigation was necessary.

Dose Selection

Dose selection was based on the data obtained from the pre-test and the highest applied dose (2000 mg/kg b.w. for 24 and 48 h) was the maximum applicable level. Two other dose groups were chosen (200 and 1000 mg/kg b.w.) at the 24 h treatment interval.

Main Experiment

For each test group five male and five female mice were assigned and tail tagged by chance. At the beginning of the experiment the animals were individually weighed and the administered volume adjusted to the animal's body weight. The animals received the test item once orally. Sampling of the bone marrow was carried out on single animals 24 and 48 hours after treatment. The animals were sacrificed by cervical dislocation. The femora were removed and the bone marrow was flushed out of the bones with 1 ml FCS (foetal calv serum). The epiphyses of the bones were then cut off and the bones were rerinsed with FCS. The bone marrow cells were then thoroughly resuspended in the FCS and then centrifuged at 1500 rpm for 10 minutes and the supernatant discarded. The pellet was then resuspended in a small volume of FCS and smeared on a slide. The smears were air dried and stained with May-Grünwald (Merck, D-64293 Darmstadt)/ Giemsa (Merck, D-64293 Darmstadt). Cover slips were mounted with EUKITT (Kindler, D-79108, Freiburg). At least one slide was made from each bone marrow sample.

Analysis of the Cells

Evaluation of the slides was performed using Olympus microscopes with 100x oil immersion objectives. 2000 polychromatic erythrocytes (PCE) were analysed per animal for micronuclei.

To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and expressed in normochromatic erythrocytes per 1000 PCEs. The analysis was carried out with coded slides. For the analysis of the cells the micronuclei was scored for small and for large micronuclei. The large micronuclei are considered to be the result of aneugenic effects, the small micronuclei are considered to be the result of clastogens (8).

Per dose group five animals of each sex were evaluated as described.

Data Recording

The generated data were recorded in the laboratory protocol. The results are presented in tables.

Evaluation of Results

A test item is classified as mutagenic if it induces either a statistically significant dose related increase in the number of micronucleated polychromatic erythrocytes or a reproducible statistically significant positive response for at least one of the test points.

A test item producing neither a statistically significant dose-related increase in the number of micronucleated PCEs nor a statistically significant and reproducible positive response at any dose levels is considered non-mutagenic in this system.

If necessary a statistical analysis can be done using the non-parametric Mann-Whitney test (6).

For the evaluation of the data both biological and statistical significance should be considered together.

Biometry

Statistical significance at the 5% level ($p < 0.05$) was evaluated by means of the non-parametric Mann-Whitney test.

Negative Control versus Test Group	Significance	p-value	
		male	female
CPA 40 mg/kg b.w. 24 hours	+	0.0079	0.0159
200 mg/kg b.w. 24 hours	-	0.5476	0.1508
1000 mg/kg b.w. 24 hours	-	0.5476	0.8413
2000 mg/kg b.w. 24 hours	-	0.4206	0.6905
2000 mg/kg b.w. 48 hours	-	0.8413	0.8413

+ = significant

- = not significant

Deviation to Project Protocol

There were no deviations to the project protocol.

Results

Pre-experiment for Toxicity

In a pre-experiment 3 females and males received a single dose of 2000 mg/kg b.w. orally. The volume administered was 20.0 ml/kg b.w..

The animals expressed no toxic reactions.

Summary of Results

Table 1: Negative Control (1 % CMC) 24 hours

Animal No.	Sex	Test Group	dose mg/kg b.w.	Micronuclei in 2000 PCE per Animal	PCE/NCE
1	M	1 % CMC	0	8	1000/601
2	M	"	0	4	1000/584
3	M	"	0	11	1000/775
4	M	"	0	5	1000/408
5	M	"	0	7	1000/1029
			Sum	35	5000/3397
			Mean	7.0	1000/679.4
Percentage of Cells with Micronuclei				**0.35**	
1	F	1 % CMC	0	2	1000/675
2	F	"	0	6	1000/637
3	F	"	0	4	1000/879
4	F	"	0	12	1000/957
5	F	"	0	2	1000/685
			Sum	26	5000/3833
			Mean	5.2	1000/766.6
Percentage of Cells with Micronuclei				**0.26**	

Table 2: Positive Control; 40 mg/kg b.w. Cyclophosphamide (CPA) 24 hours

Animal No.	Sex	Test Group	dose mg/kg b.w.	Micronuclei in 2000 PCE per Animal	PCE/NCE
1	M	CPA	40	114	1000/168
2	M	"	„	53	1000/555
3	M	"	„	22	1000/715
4	M	"	„	78	1000/540
5	M	"	„	84	1000/1062
			Sum	351	5000/3040
			Mean	70.2	1000/608
Percentage of Cells with Micronuclei				**3.51**	
1	F	CPA	40	27	1000/573
2	F	"	„	58	1000/641
3	F	"	„	65	1000/438
4	F	"	„	55	1000/974
5	F	"	„	7	1000/662
			Sum	212	5000/3288
			Mean	42.4	1000/657.6
Percentage of Cells with Micronuclei				**2.12**	

Table 3: WACKER BS 1701, 2000 mg/kg b.w. 24 hours

Animal No.	Sex	Test Group	dose mg/kg b.w.	Micronuclei in 2000 PCE per Animal	PCE/NCE
1	M	Test Item	2000	11	1000/849
2	M	"	"	5	1000/711
3	M	"	"	16	1000/557
4	M	"	"	9	1000/793
5	M	"	"	6	1000/375
			Sum	47	5000/3285
			Mean	9.4	1000/657
Percentage of Cells with Micronuclei				**0.47**	
1	F	Test Item	2000	6	1000/1084
2	F	"	"	6	1000/346
3	F	"	"	6	1000/497
4	F	"	"	3	1000/639
5	F	"	"	4	1000/640
			Sum	25	5000/3206
			Mean	5.0	1000/641.2
Percentage of Cells with Micronuclei				**0.25**	

Table 4: WACKER BS 1701, 2000 mg/kg b.w. 48 hours

Animal No.	Sex	Test Group	Dose mg/kg b.w.	Micronuclei in 2000 PCE per Animal	PCE/NCE
1	M	Test Item	2000	6	1000/742
2	M	"	"	7	1000/1082
3	M	"	"	5	1000/1844
4	M	"	"	16	1000/885
5	M	"	"	3	1000/770
			Sum	37	5000/5323
			Mean	7.4	1000/1064.6
Percentage of Cells with Micronuclei				**0.37**	
1	F	Test Item	2000	6	1000/511
2	F	"	"	2	1000/487
3	F	"	"	7	1000/522
4	F	"	"	5	1000/349
5	F	"	"	4	1000/686
			Sum	24	5000/2555
			Mean	4.8	1000/511
Percentage of Cells with Micronuclei				**0.24**	

Table 5: WACKER BS 1701, 1000 mg/kg b.w. 24 hours

Animal No.	Sex	Test Group	Dose mg/kg b.w.	Micronuclei in 2000 PCE per Animal	PCE/NCE
1	M	Test Item	1000	6	1000/863
2	M	"	"	11	1000/670
3	M	"	"	13	1000/534
4	M	"	"	4	1000/872
5	M	"	"	8	1000/343
			Sum	42	5000/3282
			Mean	8.4	1000/656.4
Percentage of Cells with Micronuclei				**0.42**	
1	F	Test Item	1000	8	1000/1014
2	F	"	"	3	1000/434
3	F	"	"	3	1000/416
4	F	"	"	5	1000/392
5	F	"	"	5	1000/802
			Sum	24	5000/3058
			Mean	4.8	1000/611.6
Percentage of Cells with Micronuclei				**0.24**	

Table 6: WACKER BS 1701; 200 mg/kg b.w. 24 hours

Animal No.	Sex	Test Group	Dose mg/kg b.w.	Micronuclei in 2000 PCE per Animal	PCE/NCE
1	M	Test Item	200	10	1000/955
2	M	"	"	10	1000/468
3	M	"	"	9	1000/1131
4	M	"	"	8	1000/496
5	M	"	"	1	1000/512
			Sum	38	5000/3562
			Mean	7.6	1000/712.4
Percentage of Cells with Micronuclei				**0.38**	
1	F	Test Item	200	7	1000/913
2	F	"	"	10	1000/653
3	F	"	"	11	1000/551
4	F	"	"	10	1000/875
5	F	"	"	8	1000/287
			Sum	46	5000/3279
			Mean	9.2	1000/655.8
Percentage of Cells with Micronuclei				**0.46**	

Discussion

The test item WACKER BS 1701 was assessed in the micronucleus assay for its potential to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the mouse.

The test item was prepared and diluted with 1 % CMC. The volume administered orally was 10 ml/kg b.w.. 24 and 48 h after a single application of the test item the bone marrow cells were collected for micronuclei analysis.

Ten animals (5 males, 5 females) per test group were evaluated for the occurrence of micronuclei. 2000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei.

To describe a cytotoxic effect due to the treatment with the test item the ratio between polychromatic and normochromatic erythrocytes (NCE) was determined in the same sample and reported as the number of NCE per 1000 PCE.

The following dose levels of the test item were investigated:

24 h preparation interval: 200, 1000 and 2000 mg/kg b.w.

48 h preparation interval: 2000 mg/kg b.w.

In a pre-experiment 2000 mg/kg b.w. was estimated to be the maximum attainable dose. At these dose levels no toxic effects were obtained after application of the test item.

The ratio of normochromatic to polychromatic erythrocytes was not affected by the treatment with WACKER BS 1701 at a dose of 2000 mg/kg b.w. at 24 hours and slightly effected after 48 treatment of the males indicating that the test item had no or only slight toxic properties.

In comparison to the corresponding negative controls there was no substantial enhancement in the percentage of cells with micronuclei at any preparation interval or dose level of the test item.

The mean values of micronuclei observed after treatment with WACKER BS 1701 (0.37-0.47 for male and 0.24-0.46% for female mice) were only slightly above the range as compared to the negative control groups (0.35% for male and 0.26% for female mice)

Statistic:

40 mg/kg b.w. cyclophosphamide administered i.p. was used as positive control which showed a distinct increase of induced micronucleus frequency (percentage of cells with micronuclei was 3.51% for male and 2.12% for female mice).

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item did not induce micronuclei as determined by the micronucleus test in the bone marrow cells of the mouse.

Distribution of the Report

Sponsor	2x (original, copy)
Study Director	1x (copy)

References

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2.Schmid,W. (1976); The micronucleus test for cytogenetic analysis; In: A. Hollaender (Ed.), Chemical Mutagens, Vol.4, Plenum Press, New York, pp. 31-53

3.Matter, B.E., and J. Grauwiler (1974); Micronuclei in mouse bone marrow cells, a simple in vivo model for the evaluation of drug induced chromosomal aberrations; Mutation Res., 23, 239-249

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